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Important role of raft aggregation in the signaling events of cold-induced platelet activation

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Abstract

When human platelets are chilled below 20 °C, they undergo cold-induced activation. We have previously shown that cold activation correlates with the main phospholipid phase transition (10–20 °C) and induces the formation of large raft aggregates. In addition, we found that the glycoprotein CD36 is selectively enriched within detergent-resistant membranes (DRMs) of cold-activated platelets and is extremely sensitive to treatment with methyl-β-cyclodextrin (MβCD). Here, we further studied the partitioning of downstream signaling molecules within the DRMs. We found that the phospholipase Cγ2 (PLCγ2) and the protein tyrosine kinase Syk do not partition exclusively within the DRMs, but their distribution is perturbed by cholesterol extraction. In addition, PLCγ2 activity increases in cold-activated cells compared to resting platelets and is entirely inhibited after treatment with MβCD. The Src-family protein tyrosine kinases Src and Lyn preferentially partition within the DRMs and are profoundly affected by removal of cholesterol. These kinases are non-redundant in cold-activation. CD36, active Lyn, along with inactive Src and PLCγ2 co-localize in small raft complexes in resting platelets. Cold-activation induces raft aggregation, resulting in changes in the activity of these proteins. These data suggest a crucial role of raft aggregation in the early events of cold-induced platelet activation.

Keywords: Raft; Signaling; Platelet; Microdomain; Protein tyrosine kinase; Cyclodextrin

1. Introduction

The lateral organization of lipids in cellular membranes, due to preferential packing, has led to the concept of lipid microdomain formation. It has been suggested that sphingolipids and cholesterol could form a ''sorting platform-like-structure'', or raft, in the lipid bilayer in which proteins could be selectively included or excluded [1]. Most of the early studies on rafts have relied heavily on the use of cold non-ionic detergent extraction and model membrane systems [2–4]. The insolubility of these membrane microdomains in non-ionic detergents such as Triton X-100 is thought to reflect the physical properties of these specific lipid/protein assemblies. Indeed, it was postulated that in membranes, rafts exist as domains in the liquid-ordered phase [2,5–7]. The high melting temperature of sphingolipids could promote phase separation and formation of liquid-ordered domains in the presence of high amount of cholesterol. Using model membranes, it was determined that only pre-existing liquid-ordered domains are insoluble in Triton X-100 [5,7]. The use of detergent extraction is now well established, and many signaling molecules have been found to be associated with these detergent-resistant membranes (DRMs) [8–11].

When platelets are chilled from 37 to 4 °C, they undergo cold-induced activation. In order to avoid this phenomenon, blood banks are forced to store human platelets at room temperature (22 °C) for only 3 to 5 days since prolonged storage often results in bacterial overgrowth [12]. To avoid any contamination, federal regulations require the destruction of all stored platelets after 5 days, which can create a chronic shortage of transfusable

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platelets [13]. In a manner similar to platelets activated by physiological agonists, chilled platelets change shape [14,15], demonstrate an increase in F-actin [16,17] and a rise in cytosolic calcium [17–20]. In addition, physiological platelet activation also induces tyrosine phosphorylation of many cytosolic proteins, including phospholipase C-γ2 (PLCγ2) and the non-receptor tyrosine kinase Syk [21,22]. Dorahy and colleagues [24,25] have noted the presence of the two non-receptor tyrosine kinases Src and Lyn in DRMs isolated from resting platelets as well as the selective partitioning of the platelet glycoprotein CD36 within this fraction. Our laboratory has previously shown that microdomains or rafts exist in human platelets at physiological temperatures and coalesce to form macrodomains during activation [26]. These events were disrupted by treatment with methyl-β-cyclodextrin (MβCD), which collapses the rafts by removing cholesterol from the plasma membrane [27,28]. The DRMs were enriched in cholesterol and sphingomyelin and showed two thermal phase transitions around 15 and 30 °C, assigned to the phospholipids and the sphingomyelin-enriched rafts, respectively [26]. To determine whether raft aggregation mediates signal transduction by clustering receptors and/or signaling molecules, we studied the partitioning of the GPI-anchored protein CD55, and the two major platelet receptors, CD36 and the integrin α_{IIb}, into DRMs isolated from cold-activated platelets. Similar to the findings of Huang et al. [23], we found that CD36 partitions within the DRMs of resting and cold-activated platelets, and is extremely sensitive to the presence of cholesterol in the plasma membrane. In contrast, the partitioning of both CD55 and the integrin α_{IIb} is not restricted to the DRMs and is not affected by the removal of cholesterol [26]. These results imply that proteins are selectively included within the DRMs and suggest an important role of raft aggregation in platelet activation.

In this study, we further investigated the function of raft aggregation in cold-induced platelet activation, determining the partitioning of downstream signaling molecules such as PLCγ2, Syk, Src and Lyn into the DRMs. While their partitioning is a selective event, independent of cell activation, their activity was found to be directly regulated by this process. Furthermore, we showed that the surface protein CD36, along with PLCγ2, Src and Lyn, is associated into raft complexes in resting platelets prior to raft aggregation and cell activation as well as in cold-activated platelets.

2. Materials and methods

2.1. Isolation of DRMs

Platelet concentrates were obtained from the Sacramento Blood Center and incubated overnight with 10 μg/ml prostaglandin E_1 (PGE_1) at 25–30 °C. Platelet-rich plasma was obtained by spinning the cells at 1400 rpm for 10 min at 30 °C. First, the cells were washed with Buffer A (100 mM NaCl, 10 mM KCl, 10 mM EGTA, 10 mM imidizole, pH 6.8) containing 10 μg/ml PGE_1 and then again with warm TKM buffer (50 mM Tris–HCl, 25 mM KCl, 5 mM MgCl and 1 mM EGTA, pH 7.2) containing 10 μg/ml PGE_1.

Washed platelets were first incubated at 37 °C with or without 10 mM MβCD for 1 h, and then they were incubated 4 °C for 1 h. Control cells were incubated for 2 h at 37 °C. Prior to fractionation, platelets were mixed with a cocktail of protease inhibitors (2 mg/ml of leupeptin (Calbiochem Novabiochem Corp., La Jolla, CA), 5 mM Pefa-Bloc (Roche Molecular Biochemicals, Indianapolis, IN), 1% aprotinin (Sigma, St. Louis, MO), 1% pepstatin A (Roche Molecular Biochemicals) and 100 nM benzamidine (Sigma)) and 2 mM of the activated tyrosine phosphatase inhibitor sodium orthovanadate (Sigma). The cells, mixed (1:1) with ice-cold 80% sucrose-TKM to obtain a final mixture of 40% sucrose, were then lysed with 1% Triton X-100 and incubated at 4 °C for 20 min. Two milliliters of cell lysates was overlaid with 6.6-ml 36% sucrose-TKM and 3.3-ml 5% sucrose-TKM and were centrifuged at $200{,}000 \times g$ for 20 h at 4 °C using a swinging bucket rotor (SW41 ti) (Beckman). Samples from the 5%, 36% sucrose-TKM and the insoluble pellet were used for analysis. The DRMs were collected at the interface of the 5% and 36% sucrose-TKM and were washed in cold PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) followed by centrifugation at $42{,}000 \times g$ for 90 min at 4 °C.

2.2. Antibodies, SDS-PAGE and Western blotting analysis

Monoclonal mouse antibody CD36 was a generous gift from Dr. Narendra N. Tandon (Otsuka America Pharmaceutical Inc., Rockville, MD). Monoclonal mouse antibodies to Lyn and Syk and polyclonal rabbit antibody to PLCγ2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas the monoclonal mouse antibody Src (clone GD11) was obtained from Upstate Biotechnology (Lake Placid, NY). For the Western blots, we used the following secondary antibodies: goat anti-mouse IgG, alkaline phosphatase conjugate (Pierce, Rockford, IL) for Syk, Lyn and Src, and goat anti-rabbit IgG, alkaline phosphatase conjugate (Pierce) for PLCγ2.

DRM fractions were mixed with reducing Laemmli buffer, boiled, separated by SDS-PAGE [29] and transferred onto PVDF (Bio-Rad Laboratories, Hercules, CA) membranes using a Bio-Rad mini trans-blot [30]. Membranes were blocked using 5% non-fat dry milk in Tween 20–TBS buffer (0.5% Tween, 10 mM Tris–base pH 7.4, 150 mM NaCl) for 1 h at 4 °C. The fractions were assayed for PLCγ2, CD36, Syk, Src or Lyn. The bound antibodies were detected by chemiluminescence (CDP-Star, Roche Molecular Biochemicals).

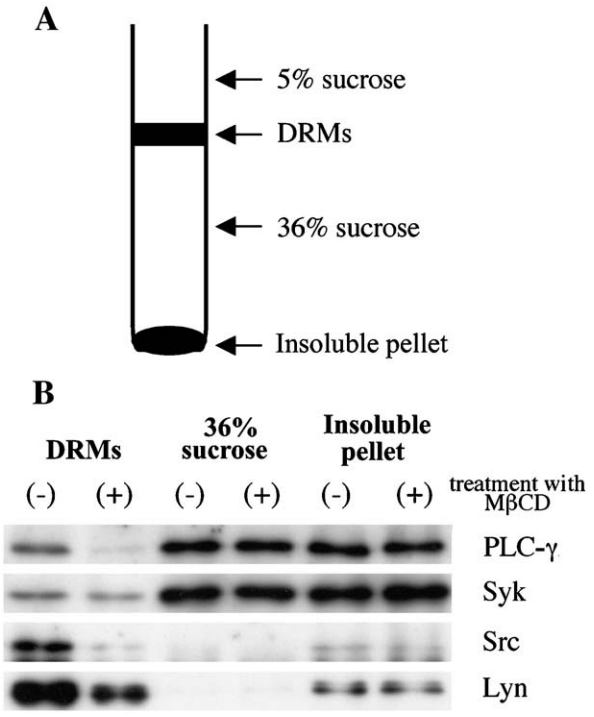

Fig. 1. PLCγ2, Syk, Src and Lyn distribution in DRMs isolated from cold-activated platelets. (A) Schematic of the sucrose density fractionations. Platelets lysed with Triton X-100 in a 40% sucrose mixture are layered with 36% and 5% sucrose buffer and centrifuged for 20 h at 200,000 × *g* (4 °C). The DRMs can be visualized between 5% and 36% sucrose interface. (B) Samples from each fraction were run on polyacrylamide gradient gels. The samples, resolved by SDS-PAGE, were transferred to PVDF membranes for Western blot analysis. No protein was detected in the 5% sucrose fraction (data not shown). Fractions were evenly loaded based on total protein content. However, the insoluble pellet cannot be broken down. Therefore, the last two lanes can only be used in a qualitative manner. Both PLCγ2 and Syk are present in all fractions, but decrease sharply in the DRMs after treatment with MβCD (61% and 28% reduction, respectively). Upon treatment with MβCD, the amount of PLC-γ and Syk only increase by 6.5% and 5.3%, respectively, in the 36% sucrose fraction. The two Src-family kinases Src and Lyn, however, are greatly enriched in DRMs, and are extremely sensitive to removal of cholesterol within this fraction. Src and Lyn are reduced by 83% and 44%, respectively, after treatment with MβCD. These results suggest that most of the proteins lost after treatment with MβCD are recovered in the insoluble pellet.

For tyrosine phosphorylation of Syk and PLCγ2, PVDF membranes were blocked with 1% BSA in low salt Tween 20–TBS buffer (0.1% Tween, 10 mM Tris–base pH 7.5, 50 mM NaCl) overnight at 4 °C. The membranes were incubated with anti-phosphotyrosine antibody conjugated to alkaline phosphatase (RC-20) (Transduction Laboratories, Lexington, KY) for 30 min at 37 °C and detected by chemiluminescence.

2.3. Immunoprecipitation and protein kinase assay

The platelets were lysed at 4 °C for 30 min in Buffer A containing 1% Triton X-100, 2 mg/ml of leupeptin (Calbiochem Novabiochem), 5 mM Pefa-Bloc (Roche Molecular Biochemicals), 1% aprotinin (Sigma), 1% pepstatin A (Roche Molecular Biochemicals), 100 nM benzamidine (Sigma) and 2 mM of activated sodium orthovanadate (Sigma). DRMs or platelets lysates from resting or cold-activated platelets (± MβCD) were pre-cleared with either mouse IgG-conjugated beads or rabbit IgG-conjugated beads (Santa Cruz Biotechnology) for 30 min at 4 °C. The beads were pelleted at 1000 × *g* for 5 min at 4 °C. The supernatants were incubated with anti-Syk-agarose-conjugated beads (Santa Cruz Biotechnology) overnight at 4 °C. For CD36 or PLCγ2 immunoprecipitation, the supernatants were first incubated with either CD36 monoclonal antibody or PLCγ2 polyclonal rabbit antibody for 1 h at 4 °C, followed by the addition of Protein-G-agarose (Calbiochem) and incubated overnight at 4 °C. For Lyn and Src, the supernatants were incubated with either Lyn or Src antibody agarose-conjugated beads (Santa Cruz Biotechnology) for 90 min at 4 °C.

For all five proteins, the beads were collected by centrifugation at 1000 × *g* for 5 min at 4 °C. The pellets were washed twice with ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mg/ml of aprotinin, leupeptin, and pepstatin, and 1 mM Na_3VO_4) and twice with ice-cold PBS. After the final wash, the pellets were resuspended in 50 μl of PBS.

Aliquots for PLCγ2, CD36, Syk, Src and Lyn were used for Western blot analysis. Aliquots for Lyn and Src were also used in a non-radioactive protein tyrosine kinase assay (Roche, Molecular Biochemicals). This photometric enzyme immunoassay uses streptavidin-coated microtiter plates, a synthetic biotin-labeled substrate peptide and a highly specific anti-phosphotyrosine antibody conjugated to peroxidase. The assay is very sensitive with a detection down

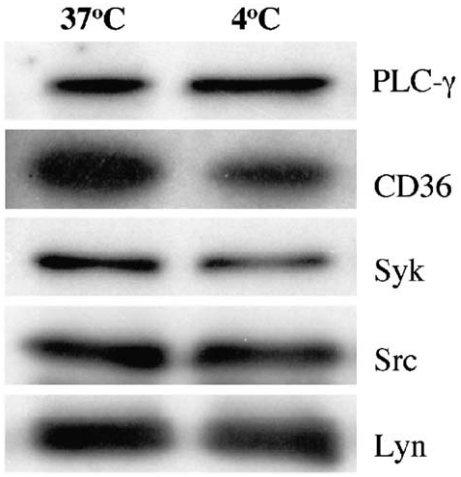

Fig. 2. PLCγ2, CD36, Syk, Src and Lyn are present in DRMs from resting and cold-activated-platelets. Western blot analysis of DRMs isolated from resting and chilled platelets shows that there is no drastic redistribution of PLCγ2, CD36, Syk, Src or Lyn after activation. PLCγ2 increases by 25% after cold-induced activation, whereas both Syk and CD36 decrease by 19% and 29%, respectively. The distribution of Src and Lyn was not affected by cell activation.

to 0.1 pmol of incorporated phosphate into the substrate. The color intensity is directly proportional to the enzyme activity and can be measured using the absorbance of the sample at 405 nm on a microtiter plate reader. The assays were run according to the manufacturer's Kit Protocol. The assay buffer contained 20 mM HEPES/NaOH, 10 mM $MgCl_2$, 3 mM $MnCl_2$, pH 7.2. The enzymes reactions were carried out for 30 min at 37 °C.

3. Results

3.1. Selective partitioning of cytosolic proteins during DRM isolation from cold-activated platelets

It has previously been shown that the partitioning of proteins into DRMs isolated from platelets using Triton X-100 was similar to that isolated with a detergent-free method [24,25]. In this study, we used detergent extraction and sucrose density gradients in order to obtain DRMs from resting or cold-activated platelets (Fig. 1A). In addition, we were able to isolate DRMs from platelets treated with MβCD and chilled to 4 °C by increasing the concentration of platelets by a factor of 5. We have previously shown that the DRMs from cold-activated platelets, found at the interface of the 5–36% interface (Fig. 1A), are highly enriched in CD36 [26]. Here we studied the partitioning of cytosolic proteins in cold-activated platelets and identified the subsequent changes in protein distribution after treatment with MβCD. Western blot analysis from the DRMs, 36% sucrose and the insoluble pellet, obtained during DRMs isolation, shows that both PLCγ2 and Syk partition into the 36% and the insoluble pellet, and are not only restricted to the DRMs (Fig. 1B). However, the partitioning of both proteins was affected by pretreating the cells with MβCD. Indeed, we observed a decrease of 61% for PLCγ2 and of 28% for Syk in the DRMs after treatment with MβCD. Contrary to what had been found

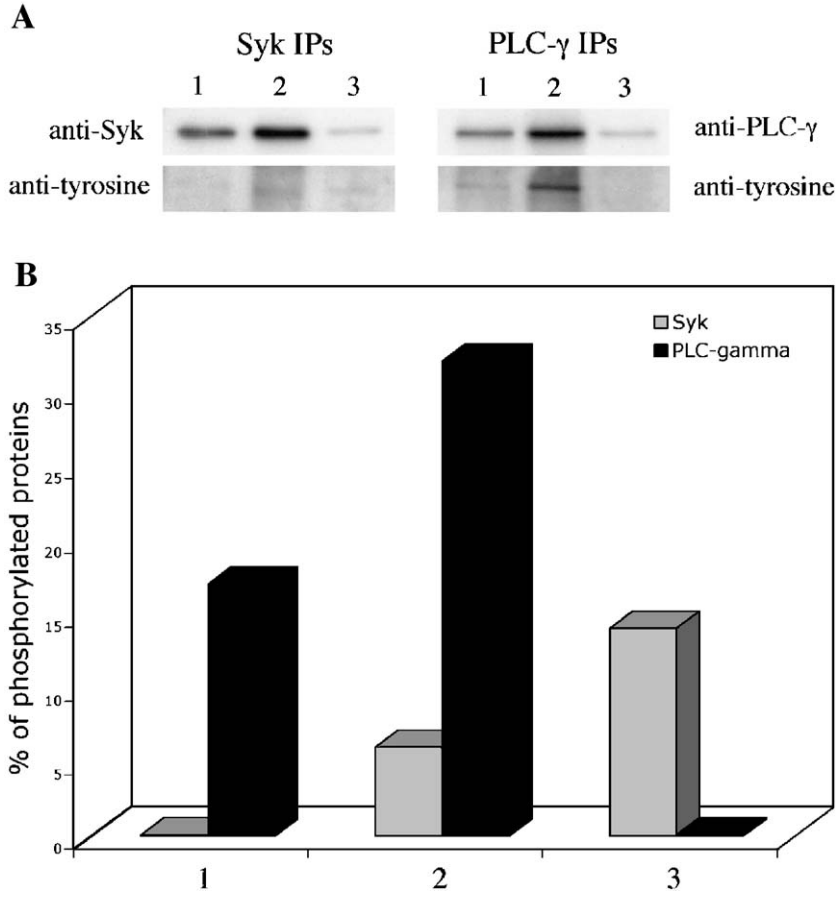

Fig. 3. Level of activity of PLCγ2 and Syk in DRMs. (A) DRMs were obtained from resting and cold-activated platelets (± MβCD). (1) DRMs of resting platelets; (2) DRMs of cold-activated platelets; (3) DRMs of cold-activated platelets pretreated with MβCD. PLCγ2 and Syk were immunoprecipitated, separated by SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were probed with anti-tyrosine antibody to determine tyrosine phosphorylation. The membranes were then stripped with β-mercaptoethanol stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 340 μl β-mercaptoethanol for 50 ml solution) for 20 min at 70 °C, and probed for PLCγ2 or Syk. Lanes (1–3) should only be compared qualitatively. (B) Percent of phosphorylated proteins based on densitometry of the Western blots for each condition. (1) In resting platelets, Syk is dephosphorylated and only 5% of the protein is phosphorylated in cold-activated platelets (2). However, almost 15% of Syk becomes phosphorylated upon removal of cholesterol (3). In cold-activated cells (2), twice as much PLCγ2 is phosphorylated compared to resting platelets (1). Disrupting the rafts with MβCD blocks PLCγ2 phosphorylation.

previously in resting platelets [24,25], our data suggest that like CD36, the two Src-related proteins Src and Lyn preferentially partition within the DRMs of cold-activated platelets (Fig. 1B). In addition, both tyrosine kinases were extremely sensitive to treatment with MβCD, with a loss of 83% for Src and 44% for Lyn from the DRM fractions after removal of cholesterol. Our data identify these four cytosolic proteins as potentially important "raft-signaling" proteins in cold-induced platelet activation.

3.2. Partitioning of proteins in DRMs isolated from resting and cold-activated platelets

If PLCγ2, Syk, Src, Lyn, along with the transmembrane receptor CD36, play an important role in cell activation via raft aggregation, then either the proteins' partitioning and/or the enzyme's activities must be different in resting versus cold-activated platelets. Since we have shown that small, individual rafts exist in resting platelets [26], we first determined whether cell activation induces a change in the protein composition of the DRMs. Fig. 2 shows that there are no drastic changes in the partitioning of the cytosolic proteins or CD36 in DRMs isolated from resting or cold-activated platelets. In chilled platelets, PLCγ2 increases by 26%, whereas both CD36 and Syk decrease by 28% and 20%, respectively. On the other hand, the partitioning of Src and Lyn was not affected by cell activation. Overall, these results show that cold-induced activation does not lead to a significant redistribution of these proteins in the DRMs.

3.3. PLCγ2 is an important 'raft signaling' molecule in cold-activated platelets

The next step was to determine the activation state of PLCγ2, Syk, Src and Lyn. Syk is not phosphorylated in resting platelets, but was found to be rapidly and transiently activated by the platelet agonist thrombin [31]. In platelets, Syk undergoes early activation in an integrin-independent mechanism, and subsequent activation as a function of integrin clustering [32]. Collagen activation results in the tyrosine phosphorylation of multiple cytosolic proteins including Syk followed by PLCγ2 [33–36]. Here, we immunoprecipitated PLCγ2 and Syk from DRMs isolated from resting and cold-activated platelets (± MβCD) and assessed tyrosine phosphorylation by Western blot analysis (Fig. 3A). The percent of phosphorylated proteins is plotted as a function of temperature for each condition (Fig. 3B). In resting platelets, Syk is dephosphorylated, whereas in cold-activated platelets, only 5% of the protein is phosphorylated. However, upon cholesterol depletion, almost 15% of Syk becomes phosphorylated. In contrast, PLCγ2 shows a very different pattern of tyrosine phosphorylation in the DRMs. Indeed, PLCγ2 is two times more active in DRMs isolated from cold-activated platelets, compared to DRMs from resting platelets. Furthermore, removal of cholesterol leads to complete inhibition of its activity, identifying PLCγ2 as an important "raft signaling" protein in cold-induced platelet activation.

3.4. Lyn tyrosine kinase is down-regulated, whereas Src is up-regulated in DRMs from cold-activated platelets

The Src-related family kinases are known to have multiple tyrosine phosphorylation sites [37–41]. They can either be repressed if phosphorylated at their C-terminus or activated if phosphorylated in their catalytic domains. Therefore, a tyrosine kinase assay is necessary to determine their activity. Falati et al. [42] have shown that Lyn is one of

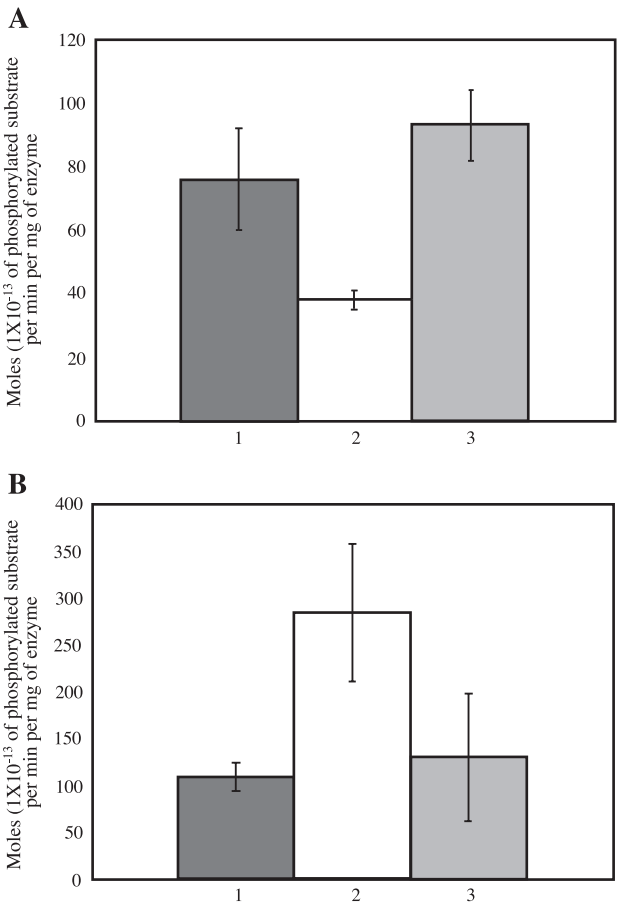

Fig. 4. Kinase activity of Src and Lyn as a function of cell activation. The activity of immunoprecipitated Lyn (A) and Src (B) from DRMs isolated from resting (1), cold-activated platelets (2) or chilled platelets pretreated with MβCD (3) were determined using a photometric enzyme immunoassay. The moles of phosphorylated substrate ($\times 10^{-13}$) per minute per milligram of enzyme was plotted for each condition. (A) The levels of activity of Lyn were similar in DRMs from resting (1) and cold-activated cells treated with MβCD (2). One milligram of Lyn phosphorylated $76 \times 10^{-13}(\pm 16)$ mol substrate/min (1), compared to $93 \times 10^{-13}(\pm 11)$ mol substrate/min (3). In cold-activated platelets, however, Lyn activity decreases by two (2). (B) On other hand, Src was found to be up-regulated in DRMs from chilled platelets, 1 mg of enzyme phosphorylating $285 \times 10^{-13}(\pm 73)$ mol substrate/min. This level of activation was over 2.5 times higher than the basal level obtained for DRMs from resting platelets ($110 \times 10^{-13} \pm 15$) or that of MβCD treated platelets ($131 \times 10^{-13} \pm 68$) (1, 3).

the Src-related kinases necessary for downstream activation of both Syk and PLCγ2 in platelets activated by von Willebrand factor. Lyn also plays a key role in collagen activation [43,44]. Finally, the kinase activity of both Lyn and Src, which is the most abundant protein tyrosine kinase in platelets [45], was altered in DRMs isolated from resting versus thrombin-activated platelets [25]. Using a photometric enzyme immunoassay, we found Lyn to be two times more active in DRMs isolated from resting cells compared to DRMs from cold-activated platelets (Fig. 4A). Upon removal of cholesterol, the activity of Lyn is restored to that of DRMs from resting platelets ($\sim 80 \times 10^{-13}$ mol phosphorylated substrate/min/mg enzyme). These results demonstrate that the activity of Lyn strongly depends on raft aggregation and that Lyn is down-regulated in cold-activated platelets. The pattern of activity for Src was opposite to that observed for Lyn. Indeed, we determined Src to be 2.6 times more active in DRMs of cold-activated cells, compared DRMs of resting platelets (Fig. 4B). Furthermore, treatment with MβCD, reduces the level of Src activity to that of DRMs from resting platelets ($\sim 100 \times 10^{-13}$ mol phosphorylated substrate/min/mg enzyme). These results demonstrate that Src is an important signaling molecule in cold-induced activation of platelets. Whereas Lyn was found to be down-regulated in chilled cells, Src is up-regulated in cold-activated platelets, demonstrating that these tyrosine kinases are not functionally redundant.

3.5. CD36, PLCγ2, Lyn and Src are part of a "raft complex"

In platelets, as well as in human melanoma and HeLa cells, Huang et al. [23] have shown that the surface receptor CD36 is physically associated with Fyn, Lyn and Yes, three Src-related tyrosine kinases. They noted that Src association to this complex depends upon the lysis buffer used during platelets' solubilization. Since we showed that CD36, PLCγ2, Lyn and Src partition into the DRMs of resting and cold-activated platelets, we further studied whether these four proteins would form a raft complex. In order to maintain the lipid–protein interactions of the plasma membrane, SDS was not added to the lysis buffer [46–48]. Similar to the platelet lysates obtained for DRMs isolation, resting and cold-activated platelets were lysed with Triton X-100, immunoprecipitated for CD36, Src and Lyn, and separated by gel electrophoresis. Western blot analysis shows that all four proteins are present in CD36-, Lyn- or Src-immunoprecipitations, independently of the activated state of the platelets (Fig. 5). These data suggest that these proteins are associated into a raft complex both in cold-activated and in resting platelets.

4. Discussion

Our data suggest that raft aggregation is a selective process, dependant upon the physical state of the membrane. In this study, we showed that PLCγ2, Src and Lyn are key signaling molecules in cold-activated platelets. Removal of cholesterol from raft domains results in profound alterations in the distribution of these three proteins.

Collagen activation of human platelets induces tyrosine phosphorylation of both PLCγ2 and Syk [37–41]. Here, we showed that tyrosine phosphorylation of PLCγ2, which is closely associated with aggregated rafts, also increases in the cold. Surprisingly, tyrosine phosphorylation of Syk, which increases in the cold, triples after the collapse of the rafts. This suggests that cholesterol directly affects the partitioning and/or the activity of cytosolic proteins. Our data identify PLCγ2 as a key molecule in the signaling pathway of cold-activated platelets, since it appears to be regulated by a "raft tyrosine kinase". On the other hand, there is an increase in Syk phosphorylation only after removal of cholesterol. In both cases, however, the fact that protein phosphorylation either drastically increased or de-

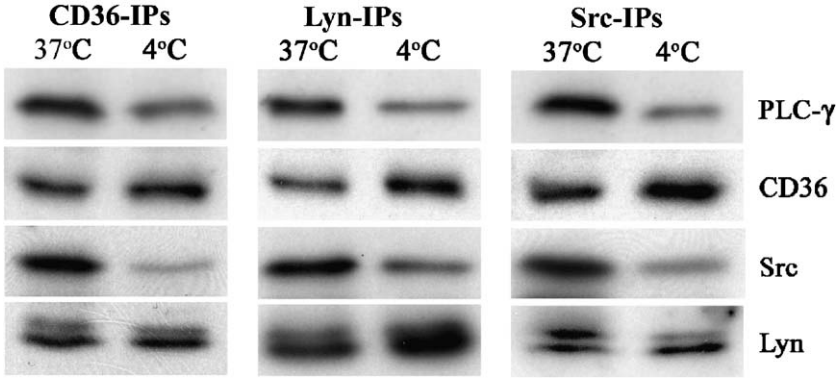

Fig. 5. Association of CD36, Src, Lyn and PLCγ2 in rafts. Platelet lysates from resting and cold-activated platelets were solubilized with Triton X-100, and immunoprecipitated for CD36, Src and Lyn. The samples were resolved by SDS-PAGE, transferred onto PVDF membranes and probed for CD36, PLCγ2, Src and Lyn. All four proteins were pulled down in all three immunoprecipitations, suggesting that they are part of a raft complex in resting, as well as in activated cells.

creased depending on the presence of aggregated rafts shows that there is a correlation between formation of a liquid-order phase and the selective partitioning of lipids and proteins into the membrane domains. The Src family-specific inhibitor PP1 has been shown to inhibit both Syk and PLCγ2 phosphorylation in platelets with activated glycoprotein Ib [42], as well as in collagen- and convulxin-activated platelets [49]. In cold-induced activation, our data suggest that kinases, such as Src, and phosphatases regulate the activity of both Syk and PLCγ2 depending on the fluidity of the membrane.

Similarly to the data of Dorahy and Burns [25] comparing resting platelets versus thrombin-activated platelets, we show here that Src is highly active in cold-activated platelets, whereas Lyn is down-regulated after chilling. Activation of proteins from the Src-related family requires the binding of proteins to their SH2 and SH3 domains, which allows for conformational changes and phosphorylation of a conserved tyrosine residue in their catalytic domain [38,41,50]. Therefore, in order to obtain the changes in the kinase activity observed during chilling, raft aggregation had to affect directly the partitioning of a number of cytosolic proteins, including adapter molecules, kinases and phosphatases. A good candidate for Lyn regulation is the Csk homologous kinase (Chk), which has been shown to regulate negatively CD36-anchored Lyn in thrombin-activated platelets by phosphorylation of Tyr508 on its carboxyl-terminal end [51]. Overall, our data clearly demonstrate that raft aggregation and partitioning of proteins within these lipid domains is a selective, dynamic process regulated by the cell and necessary for cell signaling.

Based on these results, we propose the following model for cold-induced platelet activation (Fig. 6). Small, non-functional rafts exist in resting platelets (at 37 °C). These individual rafts are too small to be visualized by conventional microscopy, but can be detected using spectroscopic methods, such as Fourier transform infrared spectroscopy [26]. CD36, active Lyn, along with inactive Src and PLCγ2 are part of raft signaling complex. Upon cold-induced platelet activation, the cells change shape, and rafts coalesce into large macrodomains, visible by fluorescence microscopy [26]. In platelets, the primary role of CD36 is still unclear. CD36 has been shown to form multimers [52], which may increase the stability of the aggregated domains. Another possible role of CD36 in raft signaling may be to induce conformational changes of associated Src-related kinases [23]. The formation of these larger, more stable domains would allow for the clustering of the signaling molecules that trigger downstream signaling events, dephosphorylation of Lyn, and phosphorylation of both PLCγ2 and Src.

Finally, cold-induced activation in platelets is associated with cell shape change [14] and correlates with an increase in cytosolic calcium [18]. Suzuki-Inoue et al. [53] have shown that activation of Src, along with phosphorylation of both Syk and PLCγ2 in platelets activated by the agonist Rhodocytin, purified from the Calloselasma Rhodostoma venom [54], requires actin polymerization. In HeLa cells as

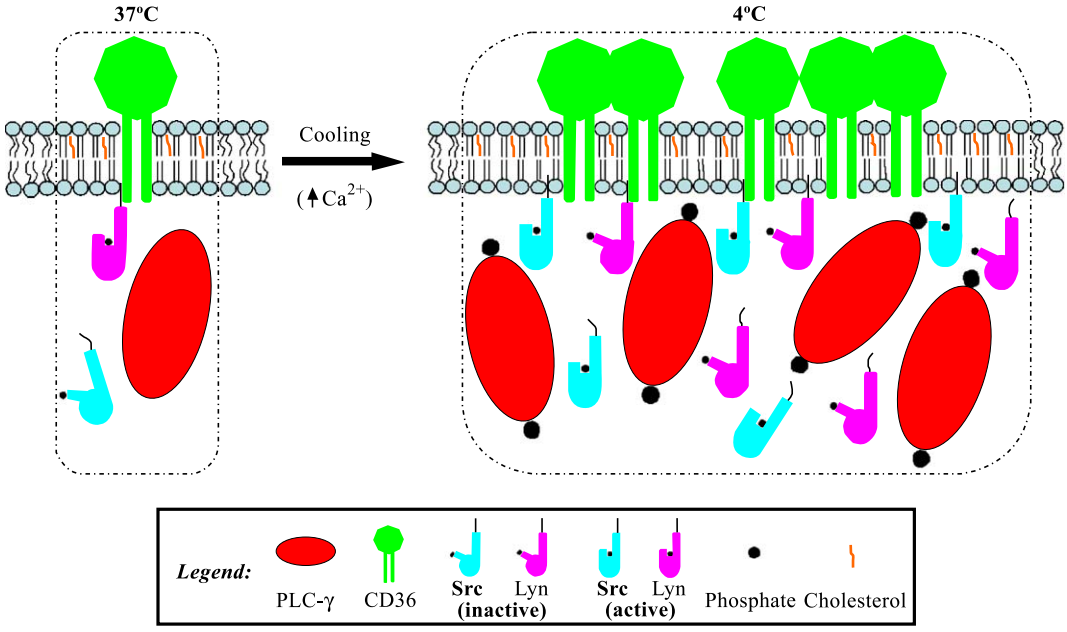

Fig. 6. Model for raft aggregation in cold-induced platelet activation. At 37 °C, rafts exist but are too small to be functional. CD36, active Lyn, and inactive Src and PLCγ2 are associated within these cholesterol-enriched microdomains. Upon chilling of the platelets through the phospholipids phase transition (~ 15 °C), platelet change shape occurs, there is an increase in cytosolic calcium, and large aggregated rafts are formed in the plasma membrane. These macrodomains allow for the clustering of signaling molecules necessary for signal transduction to occur. Raft aggregation, which could lead to CD36 oligomerization, results in the activation of Src and PLCγ2, and Lyn inactivation.

well as in stimulated T cells, Rodgers and Zavzavadjian [55] have shown that F-actin co-localizes with glycosphingolipid-enriched membrane microdomains, or raft-associated molecules, and that co-localization could be disrupted with removal of cholesterol. Furthermore, Brdickova et al. [56] have identified a cytoplasmic adapter protein that could act as a bridge between lipid rafts and the actin cytoskeleton. Overall, these studies suggest a critical relationship between membrane rafts and the cytoskeleton. Finally, similar to thrombin-activated platelets, the calcium ionophore A23187 has been shown to induce changes in platelet shape, aggregation, and secretion of the granule contents [57,58]. If the increase in cytosolic calcium observed upon cold-induced activation [18] is the result of calcium leakage from internal stores due to membrane damage during chilling, then it could also be necessary to mediate raft aggregation. Overall, these data suggest that the actin cytoskeleton and cytosolic calcium could play a significant role in raft aggregation during cold-induced platelet activation, a hypothesis that we are currently testing.

Acknowledgements

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